AD_____

GRANT NUMBER DAMD17-96-1-6104

TITLE: Role of RAC GTPase in Tumor Motility and Metastasis

PRINCIPAL INVESTIGATOR: Jie Leng, Ph.D.

CONTRACTING ORGANIZATION: Scripps Research Institute
La Jolla, California 92037

REPORT DATE: July 1998

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1998	Annual (1 Jul 97 - 30 Jun 98)

4. TITLE AND SUBTITLE
Role of RAC GTPase in Tumor Motility and Metastasis

5. FUNDING NUMBERS
DAMD17-96-1-6104

6. AUTHOR(S)
Leng, Jie, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Scripps Research Institute
La Jolla, California 92037

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The integrins regulate cell adhesion, spreading and motility in response to the extracellular matrix (ECM). Rac GTPase, one member of the Ras superfamily of small GTPases, induces membrane ruffles. We showed that adhesion activates Rac and its downstream target, p21-activated kinase (PAK), and PAK is required for integrin-mediated cell spread and migration. Rac GTPase can regulate cell migration independent of MAP kinase by reorganizing actin cytoskeleton, it also cooperates with Raf kinase to activate MAP kinase and promote motility. Using adenoviral gene delivery system, we showed that Ras induced integrin-dependent angiogenesis. These results indicate that Rac GTPase and PAK, play important roles in tumor cell motility, metastasis and angiogenesis.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	28
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

__✓__ Where copyrighted material is quoted, permission has been obtained to use such material.

__✓__ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

__✓__ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

__✓__ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Long Tu 07/29/98

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

The ability of breast cancer cells to metastasize from breast to distant organs, including the lung, brain and bone, is the major cause of death in breast cancer patients (Marx, 1993). The integrins regulate adhesion and invasion of breast cancer cells to the extracellular matrix (ECM) and form metastases at distant sites (Brooks et al., 1994). The integrins are a family of cell-surface glycoproteins that contain α and β subunits with each subunit having a large extracellular domain, a single membrane-spanning region and a short cytoplasmic domain. Integrin-mediated cell-ECM adhesion sites are complex specialized structures termed focal adhesions, at these focal adhesion sites, integrins can interact with actin filament, actin-binding protein, protein kinases and proteinases (Hynes, 1992). Actin-binding proteins that colocalize with integrins in focal contact likely impact actin filament structure, the assemblies of these structures are thought to play an important role in stabilizing cell adhesion and regulating cell shape and motility. It is also clear that integrin ligation leads to activation of a range of signal transduction events (Schwartz et al., 1995).

Ras and related GTPases are small GTP-binding proteins which regulate cell function via conversion between a GTP-bound active state and a GDP-bound inactive form. Ras proteins regulate normal and abnormal cell growth and proliferation (Bokoch et al., 1993). In this report, we show that Ras can cooperate with Rac to regulate cell motility and gene delivery of active form of Ras, RasG12V, induces angiogenesis in chick chorioallantoic membrane (CAM) assay, and its angiogenic effect is integrin-dependent.

The Rho family proteins are also members of the Ras superfamily of GTP-binding proteins including Rho, Rac and Cdc 42. It appears that one of the major roles of Rho family GTPase is to regulate actin cytoskeletal changes in normal and tumor cells (Nobes et al., 1995). They also play essential roles in Ras-inducing cell transformation (Qiu et al., 1995a and 1995b). Activation of Rac leads to the formation of lamillipodia and membrane ruffles, and Rac was implicated in cell

movement and metastasis (Ridley et al., 1992). Rac has been also shown to have a direct role in the control of cell proliferation and the activation of Jun N-terminal kinase (JNK) signaling pathway (Coso et al., 1995; Minden et al., 1995). We show Rac GTPase is required for both integrin and growth factor-mediated motility. Although Rac can activate JNK, not MAP kinase, we found it can interact with the Ras/MAP kinase pathway at the level of Raf kinase to regulate cell migration. Rac can also potentiate growth factor-induced motility which may explain why many breast cancer cells with Rac mutation are highly invasive.

One of the identified targets of Rac-GTP in cells are the p21-activated kinases (PAKs). PAKs can mediate many of the cytoskeletal changes induced by Rac and Cdc42. In this report, we show that cell adhesion can activate PAK through an integrin-dependent manner and PAK mediates cell spread and motility on ECM.

MATERIALS AND METHODS

Cells and Cell Culture

NIH3T3 and COS-7 cells were grown in Dulbecco's modified Eagle medium (DMEM) with 10 % fetal bovine serum (FBS) and 50 μg/ml gentamycin. Cells were made quiescent by maintaining them in DMEM containing 0.5% serum (NIH3T3) or serum-free DMEM (COS-7) for 24 hr.

Antibodies and Reagents

Rabbit polyclonal antibodies to Rac1, ERK2 and JNK1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibody to PAK1 was generated in this laboratory. PD98059 is a drug that specifically inhibits MEK kinase and was graciously provided by Dr. Alan Saltiel (Park-Davis, Ann Arbor, MI). Myelin basic protein (MBP) was purchased from Sigma. Collagen type I was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Fibronectin (FN) and FN 40-kDa fragment were purchased from GIBCO BRL (Gaithersburg, MD). [γ-^{32}P]-ATP was purchased from Dupont-New England Nuclear. Other chemicals and reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated.

Construction of Recombinant Adenovirus Vectors Encoding Small GTPases and Other Signaling Molecules

The adenoviral shuttle vector pAd/CI was generated by subcloning the CMV promoter-BGH polyA expression cassette from pCI vector (Promega) into a pΔE1sp1B vector (Microbix Biosystems, Toronto, Canada), using the Bgl II/BamH I sites. CDNAs encoding signaling molecules were subcloned into this vector using EcoRI/XbaI sites after PCR amplification. A portion of each construct (15 μg) and an equivalent amount of the adenovirus packaging plasmid

pJM17 (Bett et al., 1994) were cotransfected into 293 cells using calcium phosphate. The recombinant adenovirus was isolated by plague formation assay and further amplified in 293 cells as described (Bett et al., 1994).

Transfection of COS-7 Cells

COS-7 cells were transfected with lipofectamine (GIBCO) as described by the manufacturer. For each 10 cm plate, 20 µl of lipofectamine and 5 µg total DNA containing 0.5 µg of a reporter construct encoding ß-galactosidase (pCMV5-ß-galactosidase) were used. After 36 hour transfection, cells were starved in serum-free medium for 24 hours before they were tested. Cells co-transfected with ß-galactosidase were developed using X-gal as a substrate. In some cases, cells were treated with growth factor or insulin for 5 min before kinase assays, or the MEK inhibitor (PD98059, 25 µM) for 20 minutes before cell migration assays.

Immunoprecipitation and Western Blots

Cells were rinsed twice with cold PBS then lysed with RIPA buffer (100 mM Tris, pH 7.4, 0.15 M NaCl, 1% deoxycholic acid, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1% aprotinin, 2 mM PMSF, 10 µg/ml leupeptin, 5 mM EDTA, 1 mM sodium vanadate, and 50 mM NaF) for 1 hour at 4 °C. The lysate was clarified by centrifugation at 14,000 rpm for 10 minutes and the amount of total protein was determined using the BCA protein assay reagent (Pierce, Rockford, IL). The protein concentrations of the samples were normalized before either direct immunoblotting of total cell proteins or immunoprecipitation of equivalent amounts of proteins with antibodies bound to protein A sepharose beads. Samples were analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) then transferred to nitrocellulose and blocked with BSA (3%, Sigma) prior to immunoblot analysis using polyclonal or monoclonal antibodies and horseradish peroxidase conjugated goat anti-mouse or rabbit secondary antibodies and the ECL system (Amersham).

Cell Migration Assay

Cell migration assays were performed using modified Boyden chambers containing polycarbonate membranes (tissue culture treated, 6.5 mm diameter, 10 μm thickness, 8 μm pores, Transwell®; Costar, Cambridge, MA) as described previously (Klemke et al., 1994). Cells treated with the MEK kinase inhibitor PD98059 were allowed to migrate in the presence of drug for 6 hours. Migratory cells on the under surface were fixed and stained for expression of ß-galactosidase using X-gal as substrate. The number of blue cells was counted with an inverted microscope. Nonspecific or background migration was evaluated on BSA coated membranes and subtracted from all data points.

Kinase Assay

The ability of MAP kinase (ERK) to phosphorylate MBP was assayed according to Boulton et al. (1991). Briefly, 500 μg of protein from total cell lysates were precleared with protein A-Sepharose for 4 hours at 4 ^{0}C and then incubated with protein A-Sepharose coupled with anti-ERK antibodies (4 μg/100 μl stock bead suspension, Pierce) overnight in the cold. Immunoprecipitates were rinsed 3 times with RIPA and once with 0.1 M NaCl and 50 mM Hepes, pH 8.0 before incubation with 100 μl of reaction mixture containing 0.5 μCi [γ-^{32}P]ATP, 10 mM $MgCl_2$, 50 μM ATP, 1 mM dithiothreitol, 1 mM benzamidine, 0.05 mg/ml MBP, and 25 mM Hepes, pH 8.0, for 30 minutes at 30 ^{0}C. The reaction was stopped by SDS sample buffer, the reaction mixture was applied onto a 15% polyacrylamide gel. The gel was then stained with coomassie blue, dried and exposed to imaging film overnight. JNK assays were performed similarly with a little change, anti-JNK antibody was used to immunoprecipitate JNK and GST-cJun was used as the kinase substrate.

To assay PAK kinase activity, quiescent cells were plating on dishes that had been coated

with 25 μg/ml FN or FN 40-kDa fragment or anti ß1 integrin antibody. Endogenous PAK was immunoprecipitated with anti-PAK1 antibody and dissolved in SDS-sample buffer. PAK activity in the immunoprecipitates was determined using an in-gel kinase assay as described (Renshaw et al., 1996)

Angiogenesis Assay

Angiogenesis assays were performed essentially as described with minor modification (Brooks et al., 1994). Filter disc saturated with 2.0 μg/ml of bFGF or VEGF or 1.0 x 10^8 MOI of recombinant adenovirus were placed on the CAMs of 10-day-old chick embryos. 24 hr later, 50 μg of integrin antagonist LM609 (anti αvβ3) or P1F6 (anti αvβ5) was injected intravenously. After a total of 72 hr, filter discs and associated CAM tissue were harvested and quantified. Angiogenesis was assessed as the number of visible blood vessel branch points within the defined area of the filter discs.

RESULTS

Rac GTPase and PAK are activated by adhesion

Cell adhesion and spread on the ECM is mediated by integrin ligation and associated signal transduction event (Klemke et al., 1997 and 1998). Cells spread by putting out extension that contact the surface, form adhesion, and then exert tension to induce outward movement. This process is reminiscent of the actin cytoskeleton reorganization induced by Rac and Cdc42. It is likely that Rac acts as a downstream effector of integrin in regulating the actin changes associated with cell adhesion. To examine the effects of integrin ligation on Rac activity, quiescent NIH3T3 cell were plated on FN-coated dishes. Cells adhere within 5 min and spread over a period of 60 min (Figure 1). During this period, cells extend filopodia and show extensive membrane ruffles. These results suggest that Rac and Cdc42 may be activated during cell adhesion and spreading.

Direct assays of Rac or Cdc42 activation are technically difficult, therefore, we assayed the serine/threonine kinase PAK, which is the direct downstream target of these GTPases. Rac and Cdc42 interact with a number of effector proteins. The best characterized effectors are the PAKs. Activated PAK1 mutants themselves, like Rac, induce the formation of lamellipodia (Dharmawardhane et al., 1997). To minimize growth factor activation, cells were incubated for 24 hr in 0.5% serum and then detached and incubated in serum-free medium. After 3 hr in suspension, cells had minimal PAK kinase activity, but plating on FN-coated dishes strongly stimulated PAK (Figure 2). Activation was also observed when cells were plated on an antibody to ß1 integrin but not on FN-40 kDa fragment, to which cells adhere via heparin independent of integrin. These results demonstrated that integrin-dependent adhesion specifically activates PAK, by inference, Rac and /or Cdc42. Some of the above results was published this year (Leo, et al., 1998).

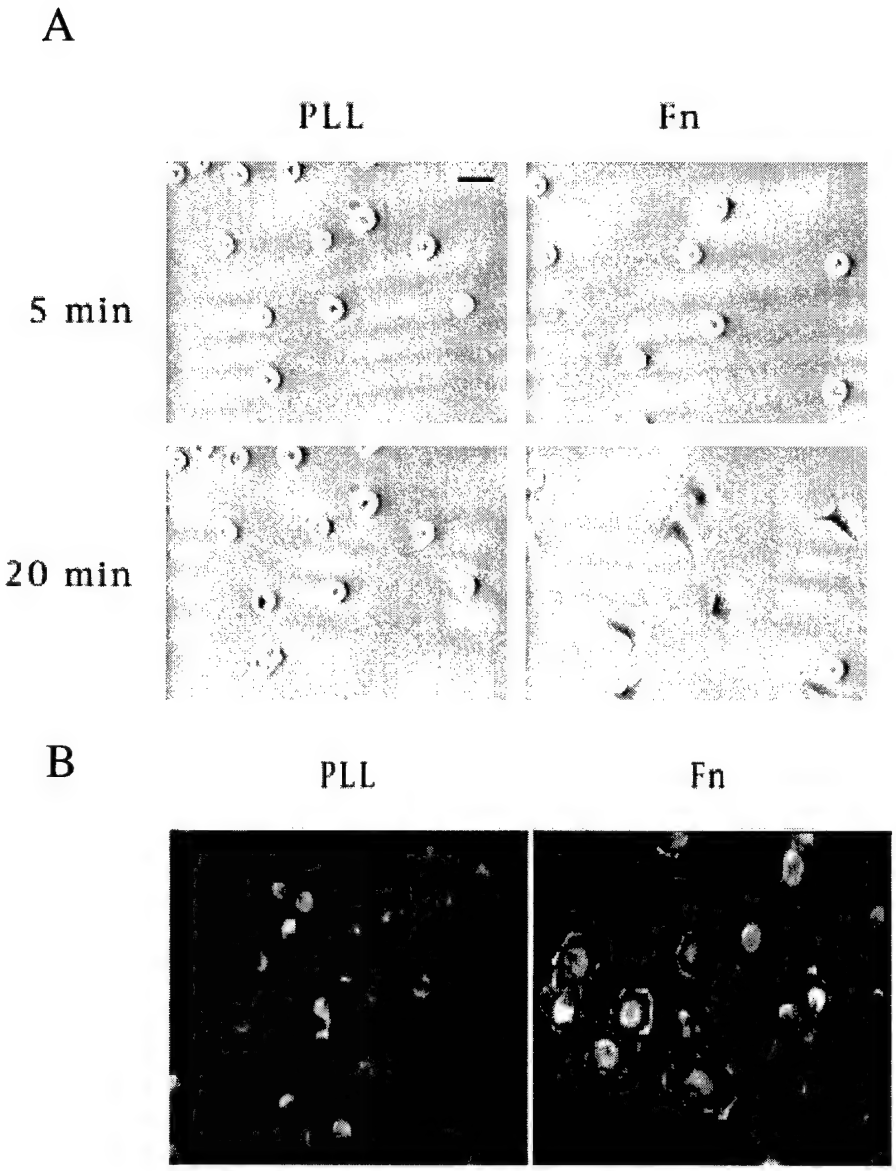

Figure 1. Cell spreading on FN and poly-L-lysine.

Quiescent cells in suspension were plated on coverslips coated with FN or poly-L-lysine (PLL). Cells were fixed at the indicated times after plating and visualized by phase contrast microscope (A). 20 minutes after plating, cells were fixed and permeablized, and actin filament was labeled with rhodamine-phalloidin. Cells were visualized by fluorescence microscopy (B).

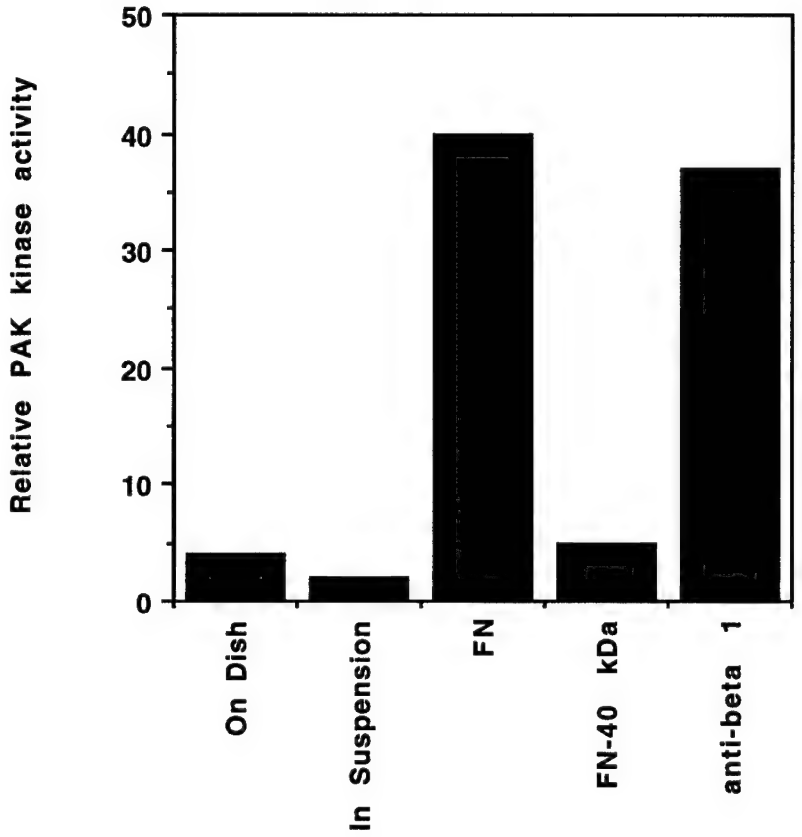

Figure 2. PAK kinase activity is stimulated after integrin-mediated cell adhesion. Quiescent cells in suspension were plated on dishes coated with FN or FN-40 kDa or anti ß1-integrin antibody for 20 minutes. PAK protein was immunoprecipitated, and its activity was assayed as described in Materials and Methods.

Rac GTPase is essential for cell migration

The Rho-family of Ras-related GTPases is required for assembly of the actin cytoskeleton and associated focal complexes (Nobes et al., 1995). Rac stimulates the formation of membrane ruffling through actin cytoskeleton reorganization. In our previous annual report (1996-1997), we showed that overexpression of dominant negative Rac, RacT17N, blocked integrin-mediated cell migration. To further investigate the role of Rac in cell motility, using a mutiwell Boyden chamber assay, expression of Rac T17N strongly inhibited cell directed migration (chemotaxis) toward EGF or insulin (Figure 3). In contrast, basal cell movement does not depend on Rac. EGF was shown to be able activate MAP kinase and induce cell migration by regulating myosin light chain kinase (Klemke et al., 1997). PD 98059 pretreated cells failed to migrate toward chemoattractants, this indicates EGF and insulin-induced chemotaxis also depends on MAP kinase. To test whether the effect of Rac is through MAP kinase pathway, MAP kinase activity assay was performed in the duplicate set of transfected cells. Figure 3B showed MAP kinase activation is not affected by Rac T17N, which suggest Rac bypass MAP kinase pathway and mediate motility mostly through controlling actin polymerization.

Rac not only can regulate growth factor and integrin-mediated cell migration, it was also shown that the induction of cell motility by the formation of adapter protein complex p130 CAS and CRK is dependent of Rac as well, and part of the results was published this year (Klemke et al., 1998). Together with our previous data, these results strongly indicate that as an important mediator of actin cytoskeleton reorganization, Rac is essential for both integrin and growth factor-mediated cell migration.

Rac GTPase potentiates EGF-induced cell migration

As we previously reported, cells overexpression of an activator of Rac, Tiam 1 (T lymphoma invasion and metastasis) have a 3-4 fold increase in cell motility on collagen. Cotransfection with a dominant negative Rac T17N blocked Tiam1 enhancement of cell motility about 70%. These results suggest that Tiam1 can promote cell migration on collagen in a Rac-

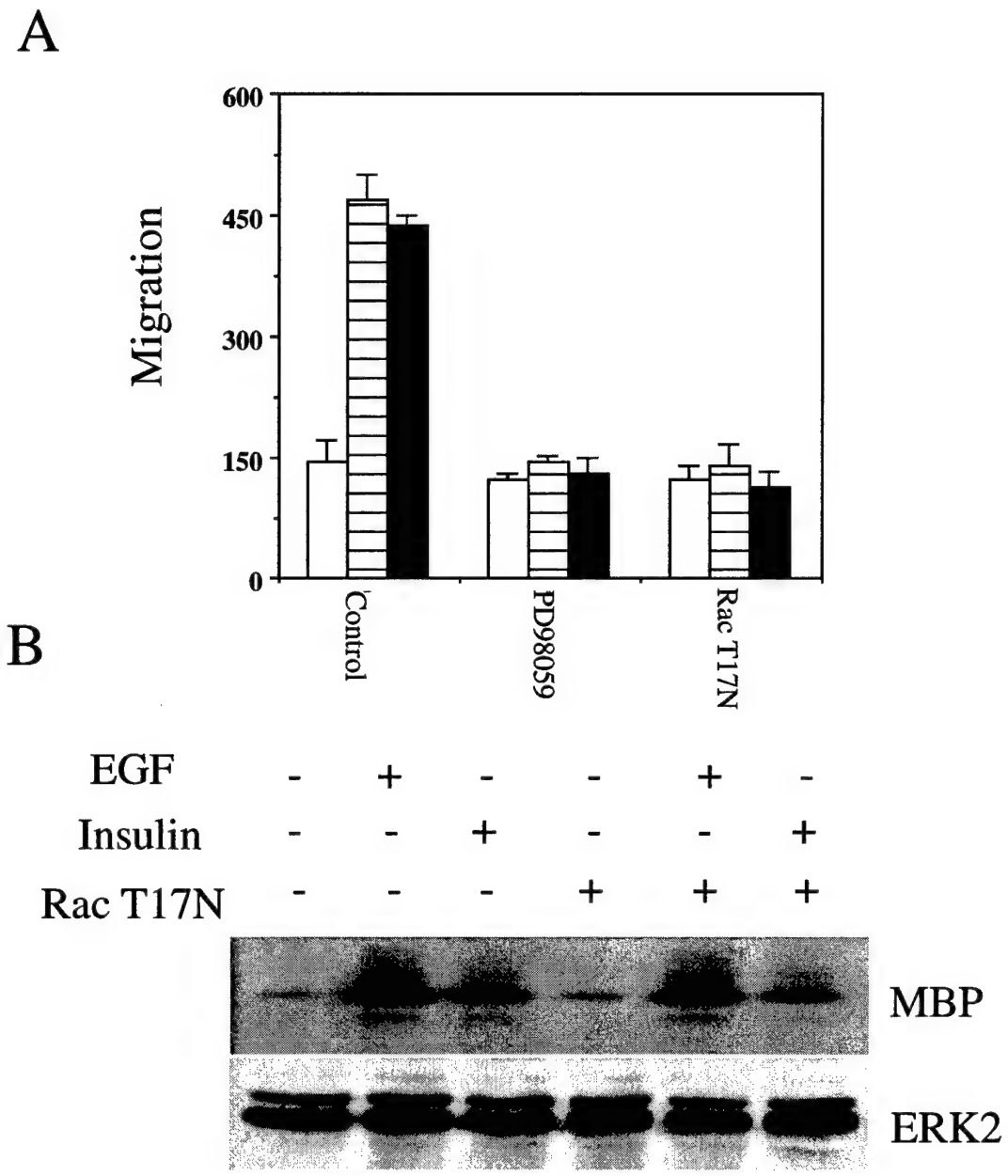

Figure 3. Rac T17N inhibits motility stimulated by EGF and insulin

COS-7 cells were serum starved for 18 hours and allowed to migrate in the absence (open bar) or presence of stimuli (hatched bar: 100 ng/ml of EGF; solid bar: 10 μg/ml of insulin) for 6 hours on collagen coated membranes after transient transfection with either the empty expression vector (control) or the expression vector containing Rac T17N. The MEK inhibitor (PD98059, 25 μM) was used to pretreat cells for 20 minutes before cell migration assays. In each case, cells were cotransfected with a ß-galactosidase containing vector for *in situ* ß galactosidase staining.

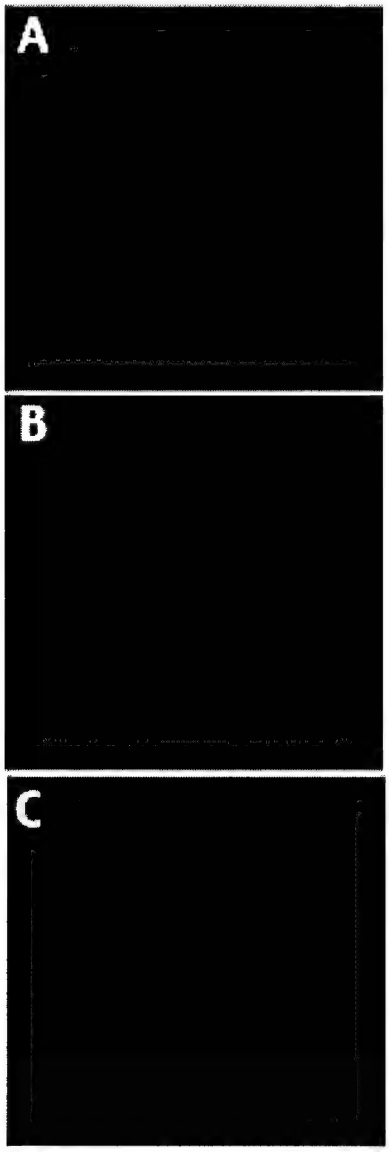

Figure 4. Rac Q61L induces cortical membrane ruffles but not polarity.
Mock (A) or Rac Q61L (B) transfected COS-7 cells were serum starved for 18 hours before they were fixed and permeablized, and actin filament was labeled with rhodamine-phalloidin. Cells were visualized by fluorescence microscopy. As a control, starved cells were treated with EGF (10 ng/ml) for 10 minutes before staining for actin filaments (C).

dependent manner. To further investigate the role of Rac in regulating cell motility, we introduced dominant active form of Rac GTPase, Rac Q61L, into COS-7 cells. Surprisingly, expression of Rac Q61L alone did not alter cell migration on collagen (Figure 5A). By examining the actin filaments, we found that unlike growth factor-treated cells, most of Rac Q61L expressing cells show extensive membrane ruffles around the entire cell body, there is no cell polarity (Figure 4). This probably explains that Rac Q61L itself alone did not promote motility. This result is somewhat different from a recent report (Keely et al., 1997), in which Rac was shown to promote epithelial T47D cell migration and invasion. To examine whether the difference could arise from different cell lines used in each study, we test the effects of Rac Q61L on cell motility of NIH3T3, 293, Hela cells as well, none of the three cell lines we tested show the enhancement of cell migration induced by Rac Q61L, therefore, we concluded that Rac is necessary but not sufficient for cell migration on collagen substrate.

Our immunofluorescent studies suggest Rac cells show extensive membrane ruffles without changing the cell polarity, EGF induces less extensive but somewhat polarized membrane ruffles at the leading edge of the cells, we reason that EGF stimulation of Rac cells should enhance cell migration in comparison with control cells. As shown in Figure 5, when cells was allowed to migrate under low dose of EGF (10 pg/ml), there is a 2-fold increase of motility over control in Rac expressing cells. Under the same concentration of EGF, mock transfected cells show no change of motility over no EGF control. Expression of Rac Q61L did not alter MAP kinase activation (Figure 5B). We also tested the EGF receptor activity, Rac also did not increase the tyrosine phosphorylation level of EGF receptor (Figure 5 C). This results suggest Rac GTPase can potentiate EGF-induced motility through a MAP kinase-independent manner. Tumor cells may activate Rac GTPase to maximize their ability to metastasize.

Interactions between Rac and Ras/MAP kinase pathway

Integrin ligation leads to the activation of focal adhesion kinase with subsequent activation

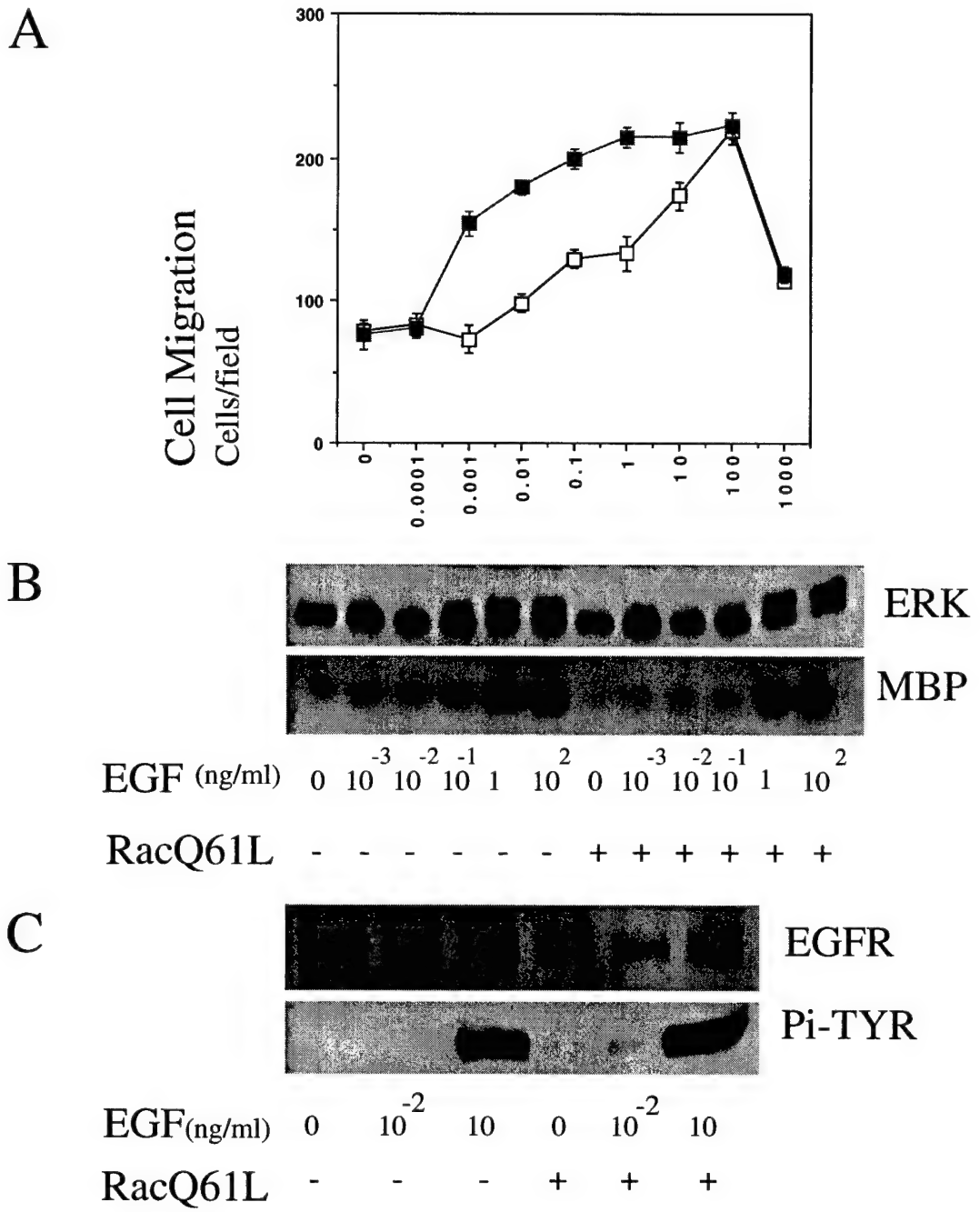

Figure 5. Rac potentiate EGF-induced chemotaxis

A: COS-7 cells were allowed to migrate toward EGF at indicated amount for 6 hours after transient transfection with either the empty expression vector (open square) or the expression vector containing Rac Q61L (close square). B and C: transfected cells were serum starved for 24 hr before treated with EGF. Cell lysates were made to assay the MAP kinase activity (B), and tyrosine phosphorylation of EGF receptor was determined by blotting with anti-phosphotyrosine antibody (C).

of Ras/MAP kinase pathway (Schlaepfer et al., 1994; Schwartz et al., 1995). Recently, MAP kinase was shown to affect cell motility by regulating the activity of myosin light chain kinase (MLCK) (Klemke et al., 1997). Although Rac activates the JNK pathway and has little effect on MAP kinase activity, it appears to play an essential role in Ras transformation (Qiu et al., 1995a). As we showed in Figure 1, both MAP kinase and Rac are required for growth factor-induced cell motility. To test this further, we showed each member of the MAP kinase cascade, including Ras, Raf and MEK, can promote Rac-depended motilities (Figure 6) . To test whether they can cooperate to regulate cell migration, we cotransfected cells with Rac and Ras or Raf or MEK. Rac can only synergize with Raf to promote motility (Figure 7A), not with Ras and MEK (data not shown). Expression of Rac together with low level of Raf can also activate MAP kinase (Figure 7B), where JNK activation induced by Rac did not changed in the presence of Raf (data not shown). PD 98059 blocked the motility and MAP kinase activation induced by Rac and Raf (Figure 7). These results suggest Rac can regulate motility at least in two way, one through regulating actin cytoskeleton which is independent of MAP kinase, one by synergizing with Raf to activate MAP kinase and MLCK.

As an direct downstream effector of Rac, PAK was shown to be able to phosphorylate MEK *in vitro*, this may explain the synergism between Rho family GTPases and Ras/MAP kinase pathway (Frost et al., 1997). To examine the role of PAK in growth factor or integrin-mediated cell migration, we microinjected a dominant negative form of PAK, Rac-binding domain of PAK (PDB) into NIH3T3 cells, PDB was shown to block integrin-mediated cell spread and movement (Leo et al., 1998), this results suggest that PAK is involved in integrin-mediated cell spread and migration.

Ras induces angiogenesis

One of the important features of tumor is its ability to release angiogenic factors and trigger signaling transductions to induce angiogenesis (Folkman et al, 1996). To examine the role of Rac and PAK in cancer cell metastasis and their ability to induce angiogenesis, we employed a CAM

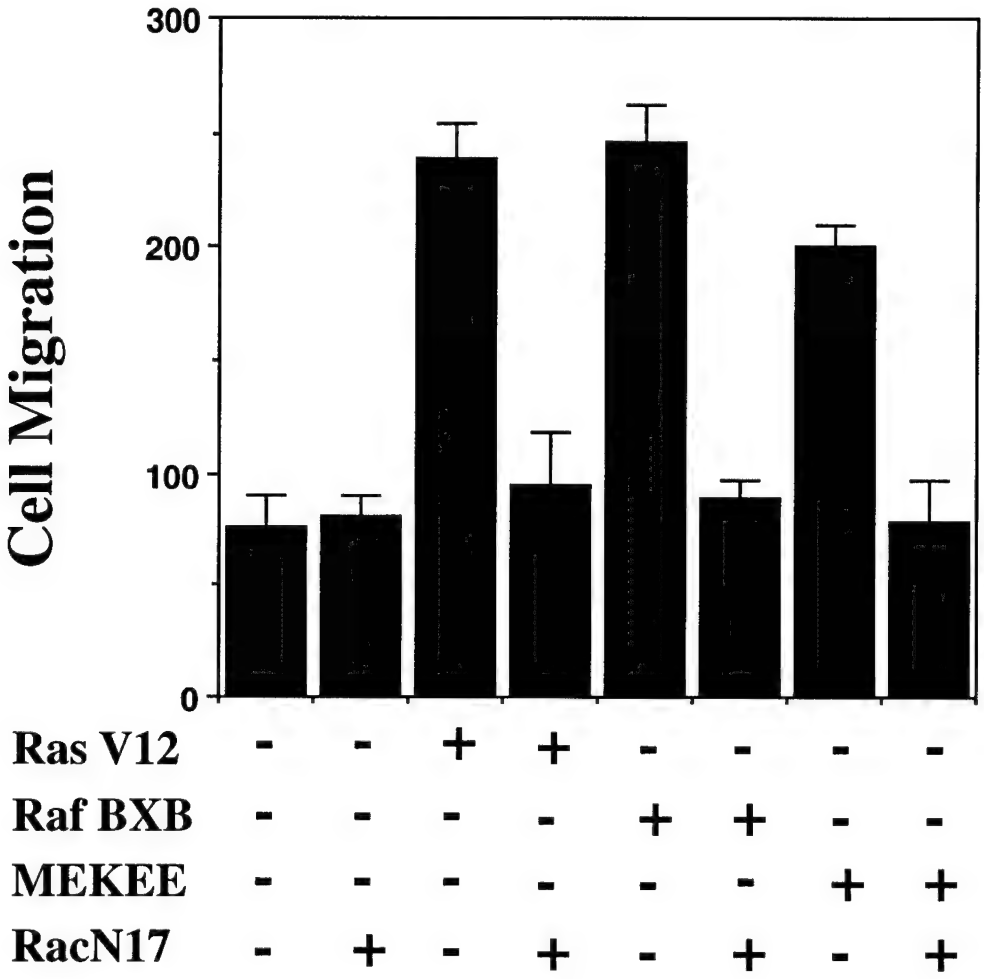

Figure 6. Rac is required for MAP kinase-mediated cell migration.
COS-7 cells were transfected with 1 μg of Ras G12V, Raf BXB or MEKEE with or without 1 μg of Rac T17N, cells were then serum starved for 18 hours and allowed to migrate for 6 hours on collagen coated membranes. In each case, cells were cotransfected with a ß-galactosidase containing vector as a reporter gene.

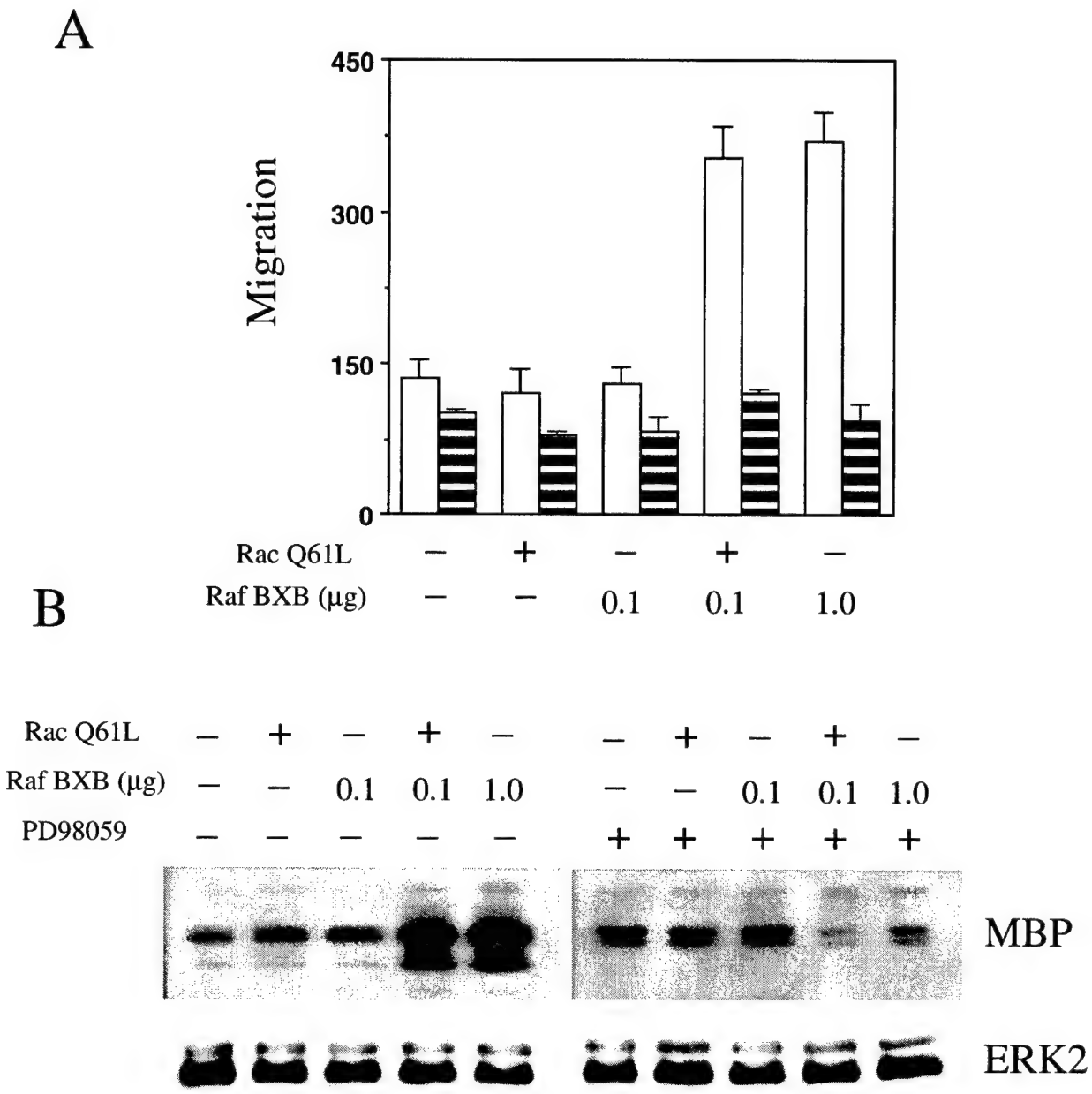

Figure 7. Rac synergizes with Raf to activate MAP kinase and promote cell migration.

COS-7 cells were transfected with 0.1 μg or 1.0 μg of Raf BXB with or without 1 μg of Rac Q61L. Cell migration (A) and kinase assays (B) were performed without (open bar) or with PD98059 pretreatment (hatched bar) as described in Materials and Methods.

model on chick embryo, since this model had been widely used to study breast tumor growth, metastasis and angiogenesis (Brooks et al., 1994). We used recombinant adenovirus to delivery signaling molecules onto CAM of chick embryos *in vivo*. Our results indicated that bFGF and VEGF-mediated angiogenesis is Ras-dependent, and active form of Ras, Ras G12V, induces angiogenesis in an integrin αvß3 and αvß5-dependent manner (Figure 8). We have successfully made recombinant viruses which express Rac mutants, PAK mutants and Ras effector mutant (data not shown). Currently, we are investigating the roles of Rac and PAK play in tumor growth and angiogenesis. Ras G12VT35S and Ras G12VY40C are two interesting effector mutants of Ras. Ras G12V activates MAP kinase pathway and Rac-mediated membrane ruffles. In contrast, Ras G12VT35S only activate MAP kinase pathway, and Ras G12VY40C only induced Rac-mediated membrane ruffles (Joneston, et al.,1996). We hope to use these two Ras mutants to dissect the downstream pathway of Ras-mediated angiogenesis. We are also starting to use MCF-7, a breast cancer cell line, test the effects of overexpression of dominant negative forms of Rac and PAK on tumor growth and tumor-induced angiogenesis in the chick CAM model.

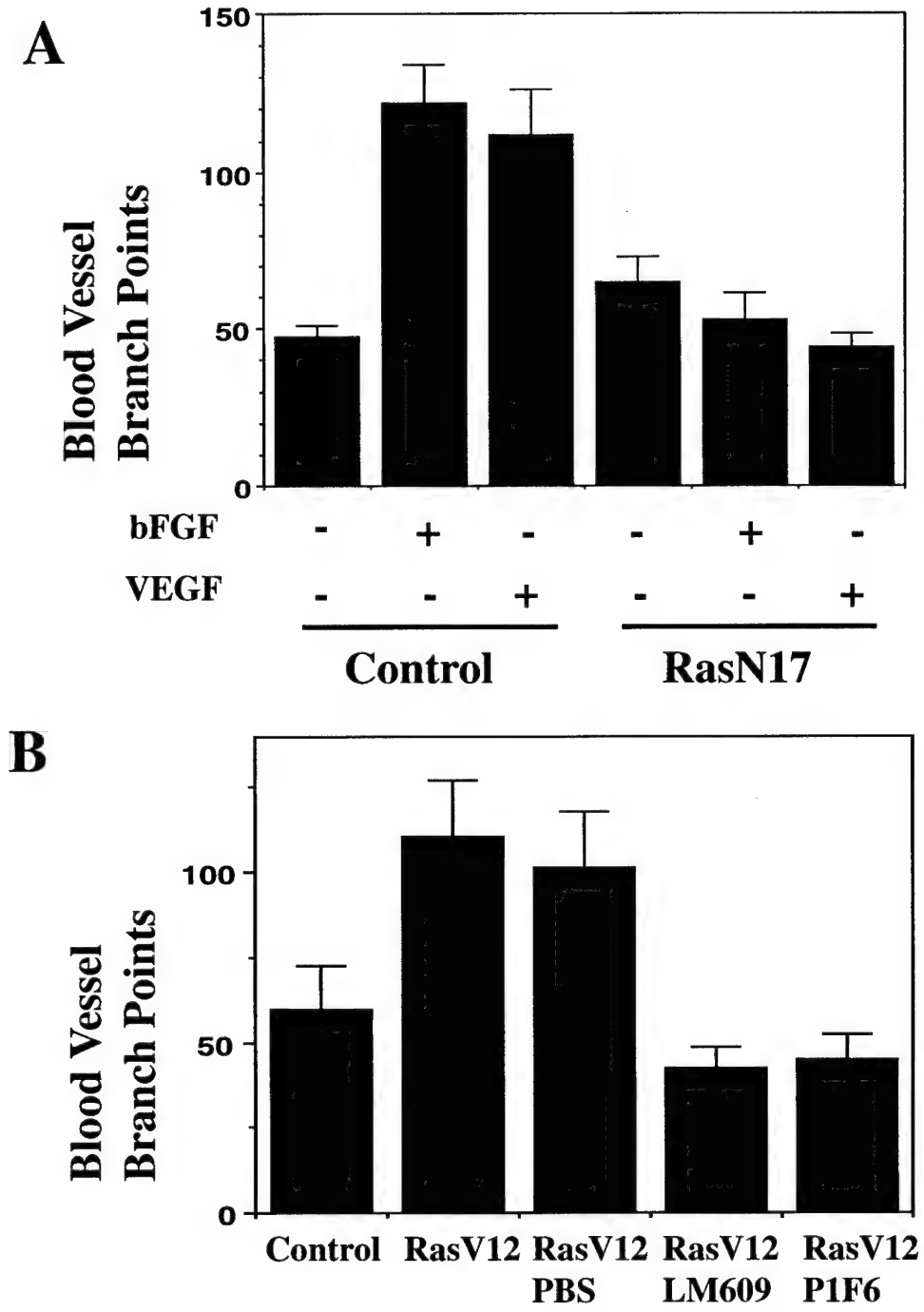

Figure 8. The roles of Ras in angiogenesis.
Filter disc saturated with 2.0 μg/ml bFGF or VEGF or 1.0 x 10^8 MOI recombinant adenovirus were placed on the CAMs of 10-day-old chick embryos. After 24 hr, 50 μg of antagonist to integrin αvβ3 (LM609) and to αvβ5 (P1F6) were intravenous injected into chick embryos, After a total of 72 hr, filter discs and associated CAM tissue were harvested an quantified.

CONCLUSIONS

Cell migration and invasion on ECM plays a critical role in tissue remodeling, tumor metastasis and angiogenesis. Integrin ligation results in the assembly of multi-molecular focal complexes associated with the actin cytoskeleton. These structures are believed to be involved in integrin-mediated signal transduction events. It has previously shown that Rac and Rho are required for the formation of these focal complexes (Nobes et al., 1995). While it is clear that redistribution of focal complexes is necessary for directional cell movement, little is known about the role of Rho family GTPase Rac, one of the major regulator of these focal complexes, on cell migration. We have now examined the role of Rac in the regulation of cell motility mediated by either integrin ligation or growth factor stimulation. When serum-starved cells were plated on polylysine or FN-coated dishes, cells placed on FN started to spread and show membrane ruffling after 15 minutes. *In vitro* kinase assays showed p21 activated kinase (PAK), one of the Rac downstream targets, was activated upon cell adhesion on the extracellular matrix. We showed PAK is required for integrin-mediated cell spread and migrations.

In this report, we provide evidence that small GTPase Rac is involved in the regulation of growth factor-mediated cell migration. Rac T17N blocks EGF or insulin-induced motility in a MAP kinase independent manner. Overexpression of an active form of Rac did not result in any significant cell migration change, suggesting perhaps that the localization of Rac is also critical for directional cell movement. In fact, we observed that cells transfected with dominant positive Rac, Rac Q61L showed extensive cortical membrane ruffling, unlike growth factor-treated cells which only show membrane ruffles at their moving edges. More importantly, we showed that at very low EGF concentration (10 pg/ml), normally, cell will not migrate toward EGF, Rac GTPase can potentiate the ability of cells to chemotaxis toward EGF. This may explain the observation that many highly metastatic breast cancer cells are having Rac mutation.

The Ras/MAP kinase pathway has been recently shown involved in the regulation of cell migration through controlling the activity of MLCK (Klemke et al., 1997). Rho has also been shown to play a role in activation of MAP kinase by adhesion on fibronectin in NIH 3T3 cells (Renshaw et al., 1996). We show here that Rac T17N blocks the motility induced by all members of the Ras/MAP kinase cascade, including Ras, Raf and MEK. On the other side, active form of Rac,Rac Q61L, can synergize with Raf to promote motility.

During the past two years under this grant, some of the studies were published in two papers of this year (Klemke et al., 1998; Leo et al., 1998). Currently, as the first author, I am writing one paper about the regulation of cell motility by Rac and PAK, and one paper about the roles of Rac and PAK play in Ras-mediated angiogenesis.

In conclusion, we found Rac and PAK activity is required for integrin or growth factor-mediated cell spread and movement. they co-operate with MAP kinase pathway to regulate actin/myosin reorganization. Using an *in vivo* gene delivery system, we are currently investigating the roles of Ras, Rac and PAK in breast tumor growth and tumor angiogenesis. Considering Rac and PAK signaling appears to be abnormal in breast cancer, this study will increase our understanding of abnormal cellular regulation in breast cancer and may lead to identification of direct therapeutic targets capable of inhibiting of tumor progression and metastasis.

REFERENCES

Bett, A.J., Haddara, W., Prevec, L., and Graham, F.L.. 1994. An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proc. Natl. Acad. Sci. USA* 91, 8802-8806.

Bokoch G. M., Der C. J. 1993. Emerging concepts in the Ras superfamily of GTP-binding proteins. *FASEB J.* 7, 750-759.

Boulton T. G., and Cobb M. H. 1991. Identification of multiple extracellular signal-regulated kinases (ERKs) with antipeptide antibodies. *Cell Regul.* 2, 357-371.

Brooks. P.C., Clark R.A. F., and Cheresh D.A.. 1994. Requirement of Integrin avb3 for angiogenesis. *Science*. 264, 569-571.

Coso O.A., Chiariello M.,Yu J., Teramoto H., Crespo P., Xu N., Miki T., and Gutkind J.S.. 1995. The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell*. 81, 1137-1146.

Dharmawardhane, S., Sanders, L.C., Martin, S.S., Daniels, R.H., and Bokoch, G.M. 1997. Localization of p21-activated kinase (PAK1) to pinocytic vesicles and cortical actin structures in stimulated cell. *J. Cell Biol.* 138: 1-14.

Dudley, D.T., Pang L., Decker S.J., Bridges A.J., and Saltiel A.R.. 1995. A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl. Acad. Sci.* 92, 7686-7689.

Folkman, J., and D'Amore, P.A. 1996. Blood vessel formation: what is its molecular basis? *Cell* 87:1153-55.

Frost, J. A., H. Steen, P. Shapiro, T. Lewis, N. Ahn, P. E. Shaw and M. H. Cobb, 1997 *EMBO J.* 16, 6426.

Hynes, R. O. 1992. Integrins: Versatility, modulation, and signaling in cell adhesion. *Cell* 69:11-25.

Joneson T., M. A. White, M. H. Wigler and D. Bar-Sagi. 1996. Stimulation of membrane ruffling and MAP kinase activation by distinct effectors of Ras. *Science* 271, 810-812.

Keely,P.J., J. K. Westwick, I. P. Whitehead, C. J. Der and L. V. Parise, 1997. Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI3K. *Nature* 390, 632.

Klemke R., Yebra. M., Bayna, E. M., and Cheresh, D. A. 1994. Receptor tyrosine kinase signaling required for integrin αvβ5-directed cell motility but not adhesion on vitronectin. *J. Cell Biol.* 127:859-66.

Klemke R. L., Cai S., Giannini A. L., Gallagher P. J., and Cheresh D. A.. 1997. Regulation of cell motility by mitogen-activated protein kinase. *J. Cell Biol.* 137, 481-492.

Klemke R. L. **Leng J.**, Molander, R., Brooks, P., Vuori, K., and Cheresh, D.A. 1998. CAS/Crk coupling serves as a molecular Switch for induction of cell migration. *J. Cell Biol.*, 140: 961-72.

Leo, P., **Leng, J.**, Schwartz, M.A., and Bokoch, G.M. 1998. Activation of Rac and Cdc42 by integrins mediates cell spreading. *Mol. Biol. Cell* 9:1863-71.

Minden A., Lin A., Claret F., Abo A., and Karin M.. 1995. Selective activation of the JNK signaling cascade and C-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. *Cell* 81, 1147-1157.

Nobes C. D. and Hall A. 1995. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with stress fibers, lamellipodia, and filopodia. *Cell* 81, 53-62.

Marx J.. 1993. Cellular changes on the route to metastasis. *Science* 259, 262-268.

Qiu R. G., Chen J., Kirn D, McCormick F. and Symons M. 1995a. An essential role for Rac in Ras transformation. *Science* 374, 457-459.

Qiu R. G., Chen J., McCormick F. and Symons M. 1995b. A role for Rho in Ras transformation. *Proc. Natl. Acad. Sci.* 92, 457-459.

Renshaw M. W., Toksoz D., and Schwartz M. A.. 1996. Involvement of the small GTPase Rho in integrin-mediated activation of mitogen-activated protein kinase. *J. Biol. Chem.* 271, 21691-21694.

Ridley A. J., Paterson H. F., Johnston C. L., Diekmann D., and Hall, A.. 1992. The small GTP-binding protein Rac regulates growth factor-induced membrane ruffling. *Cell* 70, 401-410.

Schlaepfer, D.D., Hanks S.K., Hunter T., and van der Geer P.. 1994. Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature* 372:786-791.

Schwartz, M.A., Schaller M.D., Ginsberg M.H.. 1995. Integrins: Emerging paradigms of signal transduction. *Ann. Rev. Cell Dev. Biol.* 11, 549-599.